JOURNAL OF BACTERIOLOGY, Oct. 1992, p. 6377–6385
0021-9193/92/206377-09$02.00/0

Vol. 174, No. 20

Roles of the Tn*21* *merT*, *merP*, and *merC* Gene Products in Mercury Resistance and Mercury Binding

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Received 3 June 1992/Accepted 5 August 1992

The mercury resistance (*mer*) operon of the gram-negative transposon Tn*21* encodes not only a mercuric reductase and regulatory genes but also two inner membrane proteins (MerT and MerC) and a periplasmic protein (MerP). Although the *merT*, *merP*, and *merC* genes have been implicated in Hg(II) transport, the individual roles of these genes have not been established. We created in vitro precise deletion and frameshift mutations that eliminated each of the genes singly and in combination. Our results show that both *merT* and *merP* are required for Hg(II) binding but that *merC* is not. Both *merT* and *merP* are required for full expression of Hg(II) resistance, but loss of *merP* is less deleterious than loss of *merT*. Furthermore, mutations eliminating both *merT* and *merP* decrease resistance more than the single mutations do. In contrast, mutating *merC* had no effect on Hg(II) resistance. Both the *merT* and *merP* mutations increase the threshold Hg(II) concentration for induction of *merA-lacZ* transcriptional fusions and cause an increase in the maximal expression level. In contrast, the *merC* mutation had little effect on the threshold inducing concentration of Hg(II) but decreased the level of expression. Our results show that *merT* and *merP* alone are sufficient to specify a mercury transport system. The role of *merC* remains obscure.

Plasmids conferring resistance to organic and inorganic mercury compounds are common in both gram-positive and gram-negative bacteria. Resistance to mercuric ions is conferred by mercuric reductase, which catalyzes the NAD(P)H-dependent reduction of Hg^{2+} to Hg^0, which volatilizes from the immediate environment of the bacteria (3, 32). The most-studied gram-negative mercury resistance (*mer*) operons are from Tn*501* (from the *Pseudomonas aeruginosa* plasmid pVS1) and Tn*21* (from the *Shigella* plasmid R100, which is identical to NR1 and R222) (Fig. 1). The DNA sequences of both operons (2, 4, 5, 33, 34) reveal several open reading frames in addition to *merA*, which encodes the reductase. All of these reading frames have been correlated with protein products (2, 25, 37).

Two *mer* proteins have regulatory functions. The *merR* gene product binds the promoter-operator region, where it both positively and negatively regulates the expression of the divergently transcribed structural genes and also negatively regulates its own expression (10, 11, 15, 27, 41, 45, 48). The purified *merD* gene product also binds the operator-promoter region, albeit very weakly (35), and mutants lacking *merD* show increased operon expression, suggesting a role for *merD* in downregulation (40). Mutations deleting *merD* have, however, only a slight effect on Hg(II) resistance in multicopy plasmids (5, 25, 38).

Several lines of evidence suggest that the remaining genes, *merT*, *merP*, and *merC*, specify an Hg(II)-specific transport system. (i) The *merT* and *merC* gene products are cytoplasmic membrane proteins (18), and at least 50% of the *merP* gene product is located in the periplasm (2). (ii) Deletion and insertion mutations in *merA* confer hypersensitivity to Hg(II); i.e., the mutants are more sensitive to Hg(II) than are isogenic strains lacking *mer*. This hypersensitivity is dependent upon the presence of at least some of the *merTPC* region (2, 36, 38). (iii) The hypersensitive mutants also exhibit Hg(II)-inducible Hg(II) binding (1, 36, 38). (iv) Deletion of *merT* and *merP* genes from Tn*501* leads to an almost complete loss of the Hg(II)-resistant phenotype, and expression of Tn*501* *merT* and *merP* in the absence of mercuric reductase produces an Hg(II)-supersensitive phenotype (26). (v) A *merC* homolog from an Hg(II)-resistant strain of *Thiobacillus ferrooxidans* mediates Hg(II) binding when expressed constitutively from a multicopy plasmid in *Escherichia coli*, although the extent of binding is less than that mediated by the *merT-merP* system of the broad-spectrum mercury resistance plasmid pDU1358 from *Serratia marcescens* (24). The current model for Hg(II) binding mediated by Tn*21* and Tn*501* proposes that MerP in the periplasm initially binds Hg(II) and transfers it to MerT (and possibly MerC), which in turn transfers Hg(II) to the cytoplasm or directly to the reductase (3, 32, 34).

Although the *merT* and *merP* gene products have been implicated in Hg(II) transport, the effect of their absence on *mer* operon function in strains that have the wild-type *mer* promoter and other structural genes intact has not been reported, nor have their individual roles been examined. The role of *merC* is particularly unclear. Although the *merC* gene from *T. ferrooxidans* appears to encode an Hg(II) transport protein (24), Tn*501*, which is fully Hg(II) resistant, lacks a *merC* gene, indicating that *merC* is not essential for Hg(II) resistance. Barrineau and Summers, however, suggested that the *merC* region might also play a regulatory role in operon expression since Tn*5* and Tn*3* insertions mapping in or near Tn*21* *merC* failed to synthesize any Hg(II)-inducible *mer* polypeptides (1).

The aim of the present study was to define precisely the roles of MerT, MerP, and MerC of Tn*21* in Hg(II) resistance,

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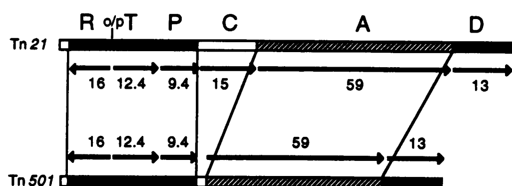

FIG. 1. *mer* operons of Tn*21* and Tn*501*. Shading indicates the degree of homology between the two operons: black, >80%; hatched, 60 to 70%; open, none. Arrows show the predicted polypeptides, and o/p indicates the divergent operator-promoter region. The numbers above or below arrows are the sizes (in kilodaltons) of associated polypeptide gene products.

Hg(II) binding, and operon induction. Because no previously described Tn*21 mer* mutations affected only the transport functions, we constructed in vitro precise deletion and frameshift mutations that eliminated each of the *merTPC* genes singly and in combination while leaving the other genes intact and expressed from the *mer* promoter.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids are shown in Table 1. The constructions of plasmids pNH6, pNH8, pNH9, and pNH10 are shown in Fig. 2.

Media. Bacteria were routinely grown in LB medium (31). L agar consisted of LB medium plus 15 g of Bacto-Agar (Difco) per liter. When appropriate, antibiotics (Sigma Chemical Co.) were added at the following concentrations: ampicillin, 50 μg/ml; tetracycline, 12.5 μg/ml; chloramphenicol, 30 μg/ml; nalidixic acid, 20 μg/ml; and kanamycin, 50 μg/ml. Tryptone broth (10 g of Bacto-Tryptone [Difco] and 5 g of NaCl per liter) was used for Hg(II) induction and Hg(II)-binding studies. Minimal A glucose plates containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml (31) were used to screen for β-galactosidase activity.

Enzymes and reagents. Restriction enzymes, T4 DNA ligase, and DNA polymerase (large fragment) were obtained from New England BioLabs, Inc., Boehringer Mannheim Biochemicals, or International Biotechnologies, Inc., and used as specified by Maniatis et al. (28) or the manufacturer. Radiochemicals were obtained from Amersham or DuPont NEN Research Products. NuSieve and SeaPlaque agarose were obtained from FMC Corp.

DNA methods. Isolation of plasmid DNA by the alkaline lysis method, agarose gel electrophoresis of DNA, filling of restriction fragments with protruding 5′ ends, ligation of DNA fragments, and transformation of *E. coli* by the calcium chloride method were carried out as described by Maniatis et al. (28). Individual restriction fragments were isolated either on DEAE-cellulose by the method of Dretzen et al. (7) or in low-melting-temperature agarose by the method of Struhl (46).

Mutant construction. All mutations were originally constructed in pNH6. Because the restriction enzymes used to create the mutations typically cleaved at multiple sites in pNH6, various approaches were used in mutant construction; two examples are shown in Fig. 3. Typically, we isolated unique restriction fragments containing only the desired sites, digested the isolated fragments, and then ligated them to the portion of pNH6 containing the replicon and the remainder of the *mer* operon. For each mutant, we tested the Hg(II)-resistance phenotype of several independent transformants, verified the predicted restriction sites, assayed Hg(II) reductase activity, and determined the presence or absence of MerP by nondenaturing polyacrylamide gel electrophoresis (PAGE).

The locations of the mutations are summarized in Fig. 4. The ΔT16 mutation was constructed by joining the *Nae*I site at bp 643 (base pair numbering as in Barrineau et al. [2]) to the cleaved and filled *Ava*II site at bp 907. The fsP1 mutation was constructed by cleaving, filling, and rejoining the *Nhe*I site at bp 1042. The ΔC37 mutation was formed by joining the cleaved and filled *Bss*HII site at bp 1313 to the filled and cleaved *Ava*II site at bp 1664. In the ΔTP37 mutation, the *Nae*I sites at bp 643 and 1217 were joined. The ΔPC1 mutation had the cleaved and filled *Nhe*I site at bp 1042 joined to the cleaved and filled *Ava*II site at bp 1664. In the ΔTPC18 mutation, the *Nae*I sites at bp 643 and 1647 were fused.

Assessment of mutant phenotypes. Mercury sensitivity was assessed by efficiency of plating (EOP) of fresh overnight cultures grown in LB (plus antibiotics as appropriate) on various concentrations (usually 50 μM) of $HgCl_2$ in L agar (1). For strains containing pNH8/pNH9 derivatives, the EOP was determined by plating on L agar containing ampicillin

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype[a]	Source or reference
E. coli strains		
JM103	Δ(*lac pro*) *thi strA supE endA sbcB hsdR* F′*traD36 proAB lacI*^{q}*Z*Δ*M15*	Messing et al. (30)
DU1040	*his lac rpsL nalA*	Foster (8)
MC1061-5	*araD139* Δ(*ara leu*)*7697* Δ*lacX74 galU galK* r_K^- m_K^+ *strA*	Koop et al. (23)
Plasmids		
pTZ19R	Ap^r *lacZ′* F1 ori pUC19 polylinker	Pharmacia
pFZY1	Ap^r p_{lac}^- *lacZ lacY lacA′ oriF* M13mp18 polylinker	Koop et al. (23)
pDU202	Cm^r Sm^r Su^r Hg^r (Tet^s derivative of R100)	Foster and Willetts (13)
pDB7	Tc^r Hg^r	Barrineau et al. (2)
pDG125	Ap^r *merR* colE1 replicon	Gambill (13a)
pACYC177	Ap^r Km^r P15A replicon	Chang and Cohen (6)
pNH6	Ap^r Hg^r (*merTPCAD*) colE1 replicon	This study
pNH8	Ap^r *merTPCAD oriF*	This study
pNH9	Km^r *merR* P15A replicon	This study
pNH10	Ap^r *merTPC lacZY oriF*	This study

[a] Plasmid designations are those of Novick et al. (39).

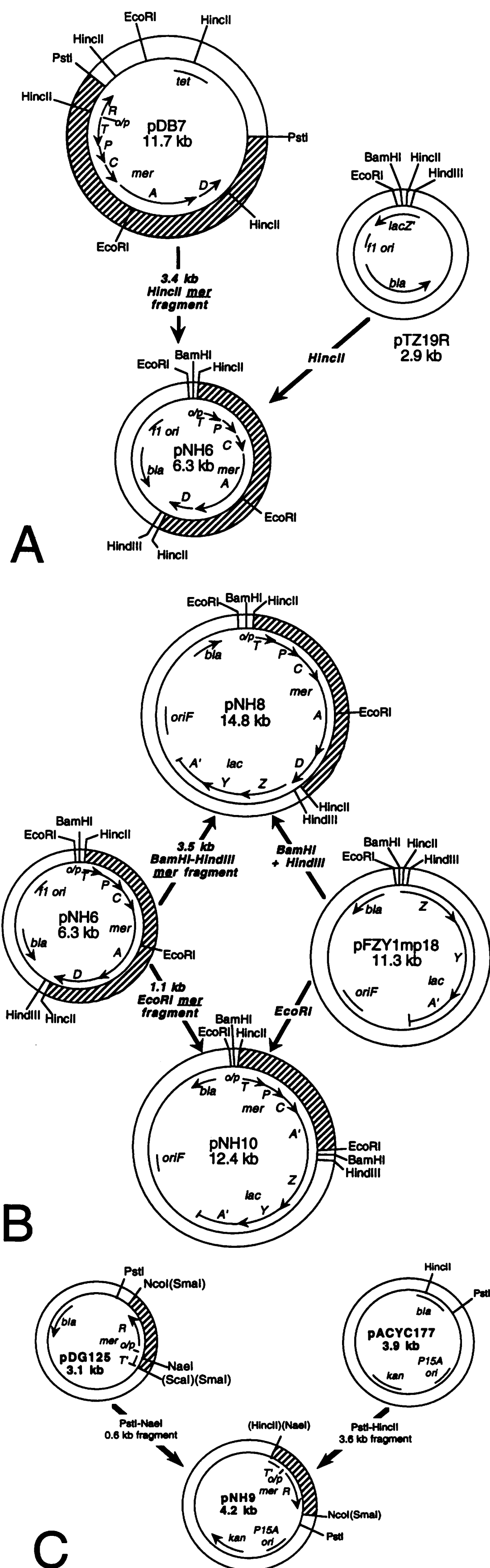

FIG. 2. Construction of (A) the multicopy *merTPCAD* plasmid pNH6, (B) the monocopy *mer* derivatives pNH8 and pNH10, and (C) the MerR-providing P15A-based plasmid pNH9. Cross-hatched segments correspond to *mer* genes, and o/p indicates the *mer* operator-promoter region.

and kanamycin and on L agar containing ampicillin, kanamycin, and $HgCl_2$.

For assays of Hg(II) reductase, Hg(II) binding, and operon induction, tryptone broth cultures were inoculated with 0.01 volume of an overnight broth culture and then grown at 37°C with vigorous shaking to 70 to 80 Klett units (green filter) or an optical density at 550 nm of 0.5. Induction, if any, was carried out as indicated below for individual experiments.

For Hg(II) reductase assays, cells were harvested by centrifugation, washed with Minimal A Medium (31) with sodium citrate omitted and resuspended in a buffer containing 50 mM sodium phosphate, 5 mM $MgCl_2$, and 2 mM 2-mercaptoethanol (pH 7.4). Cells were lysed at 12,000 lb/in^2 in a French pressure cell, and particulate material was removed by centrifugation for 45 min at 45,000 × *g*. Reductase activity in the supernatant was assayed by the disappearance of ^{203}Hg (5×10^4 cpm/nmol; 10 μM initial concentration) as described by Summers and Kight-Olliff (49), except that the reaction contained 50 mM phosphate buffer (pH 7.4), 0.2 mM $MgCl_2$, 0.1 mM 2-mercaptoethanol, 5 mM EDTA, and 100 μM NADH.

Hg(II) binding by cells was measured with 2 μM $^{203}Hg^{2+}$ (5×10^4 cpm/nmol) in tryptone broth by membrane filtration as described by Nakahara et al. (36), except that EDTA and $MgSO_4$ were omitted from the incubation mixture and polysulfone membranes (Schleicher and Schuell or Gelman) were used for filtration. Chloramphenicol (30 μg/ml) prevented induction during the binding assays. Cultures used for binding studies were inoculated from freshly streaked plates.

Operon induction was assessed with *merA-lacZ* transcriptional fusions (pNH10, Fig. 2B). Cultures were induced by transferring aliquots to tubes or flasks containing sufficient $HgCl_2$ to produce the indicated final concentration. After a 30-min induction, the cultures were placed on ice and then assayed for β-galactosidase by the method of Miller (31).

Visualization of MerP protein by native PAGE. Native polyacrylamide gel electrophoresis was carried out with cationic buffer system 1193 of the Jovin output (19–22), which stacks at pH 7.4 and separates at pH 7.0 at 25°C. The system components are as follows: the upper buffer contained 0.040 M Bis-Tris [2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol] and 0.025 M Tricine [*N*-tris(hydroxymethyl) methyl-glycine], the stacking buffer contained 0.044 M Tris and 0.028 M Tricine, the separating buffer contained 0.096 M KOH and 0.217 M Tricine, and the lower buffer contained 0.050 M KOH and 0.062 M Tricine. The separating gradient polyacrylamide gel was 18% T and 2.6% C. The stacking gel was 3.5% T and 2.6% C.

Samples for electrophoresis were prepared from bacteria grown overnight in LB plus antibiotics. Three milliliters of the cultures was centrifuged, the pellet was resuspended in 23 μl of lysing solution (7 M urea, 20% Triton X-100), and 2 μl of DNase I (10 mg/ml) was added. After 5 min at room temperature, 20 μl of this extract was loaded onto the gel. Purified MerP protein (24a) was loaded at 4 μg per well.

Electrophoresis was carried out at 200 V constant voltage. After electrophoresis, gels were fixed in 20% trichloroacetic acid and then silver stained (29).

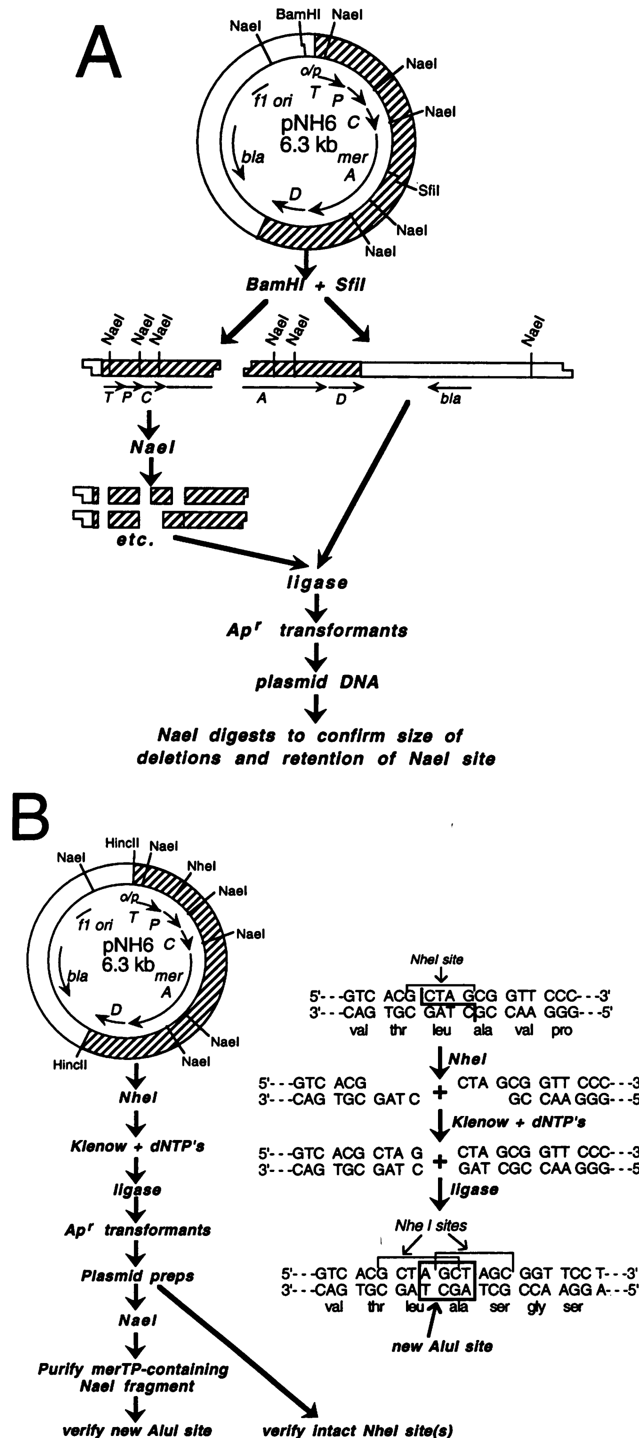

FIG. 3. Construction of (A) the ΔTP and ΔTPC mutants and (B) the fsP mutant.

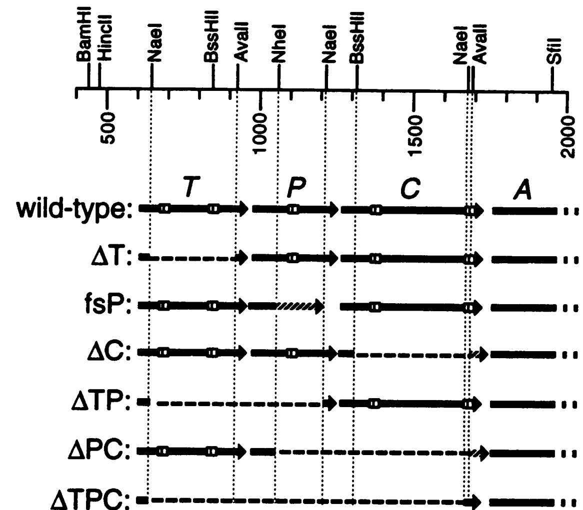

FIG. 4. Deletion and frameshift mutations in the *merTPC* region. Arrows show the predicted polypeptides. Deletions are indicated by dashed lines, and frame-shifted peptides are represented by diagonal cross-hatching. Boxes show cysteine residues. Base pair numbers are as in Barrineau et al. (2).

RESULTS

Properties of pNH6. The parent *mer* plasmid, pNH6, used for mutant construction has several useful properties. The pTZ19R phagemid vector on which it is based permits production of single-stranded DNA for mutagenesis and sequencing without subcloning into M13, and its high copy number facilitates preparation of large quantities of DNA. Insertion of the *mer* fragment into the pTZ19R multiple-cloning site created a *mer* cassette so that the *mer* mutants could easily be transplanted into other plasmid backgrounds. *merR* was deleted so that effects of alterations in Hg(II) transport on mercury resistance per se could be examined independently of effects on operon induction.

In the low-copy-number *mer* plasmid R100, *merR* mutants express mercuric reductase microconstitutively and have a mercury-sensitive phenotype (12), since the *merR* gene product is required for induction as well as repression. In the high-copy-number pTZ19R background, however, micro-constitutive expression results in mercuric reductase activity (Table 2) and mercury resistance (Table 3) that are nearly as great as those of the wild-type plasmid pDU202. Thus, pNH6 allowed assessment of the mercury resistance phenotype and of polarity effects in the same background used for mutant construction.

***merTPC* mutations.** Since the complete sequence of the Tn*21 mer* operon is known (2, 4, 5, 33, 34), we were able to create our mutations in vitro by manipulating strategically located restriction sites. The mutations were designed to eliminate as much of the gene product as possible, especially the cysteine residues believed to be important in mercury

TABLE 2. Constitutive mercury volatilization by pNH6

Plasmid[a]	Mercury volatilization (nmol of Hg · min^{-1} · mg of protein^{-1})[b]		n
	Uninduced cells	Hg-induced[c] cells	
pDU202	0.12 ± 0.06	7.14 ± 3.54	3
pNH6	5.69 ± 0.67	5.00 ± 0.99	3
pTZ19R	0.21 ± 0.01	0.17 ± 0.11	2

[a] All in *E. coli* JM103, except that one trial of pDU202 was in SK1592.
[b] Initial rates of ^{203}Hg volatilization from cell suspensions containing 10 μM $^{203}HgCl_2$. Values are means ± standard errors of the means.
[c] Cells were induced by adding $HgCl_2$ to a final concentration of 5 μM at 15 and 30 min before the cells were harvested.

TABLE 3. Mercury resistance of pNH6 and mutant derivatives

Plasmid[a]	EOP on 50 μM $HgCl_2$		
	Mean[b]	95% confidence interval[b]	n
None	1×10^{-6}	0.8×10^{-6}–3×10^{-6}	6
pTZ19R	1×10^{-6}	0.6×10^{-6}–2×10^{-6}	6
pNH6	0.67	0.39–1.1	7
pNH6ΔT	3×10^{-6}	0.6×10^{-6}–16×10^{-6}	3
pNH6fsP	4×10^{-4}	3×10^{-4}–8×10^{-4}	6
pNH6ΔC	0.64	0.31–1.3	4
pNH6ΔTP	2×10^{-6}	1×10^{-6}–4×10^{-6}	5
pNH6ΔPC	1×10^{-4}	0.6×10^{-4}–3×10^{-4}	3
pNH6ΔTPC	1×10^{-6}	0.8×10^{-6}–3×10^{-6}	6
pDU202[c]	0.79	0.46–1.3	7

[a] All in *E. coli* JM103.
[b] Calculated from log-transformed data.
[c] Tet[s] derivative of R100.

binding (3, 32), while having the mutant polypeptide terminate near its wild-type termination site to minimize polar effects on the downstream genes (Fig. 4).

Properties of *merT*, *merP*, and *merC* mutants in a multicopy, constitutive background. Both *merP* and *merT* were clearly required for normal efficiency of plating on 50 μM Hg(II) (Table 3). Loss of *merT* had a more drastic effect, decreasing the EOP by nearly 10^6-fold. Loss of *merP* caused a less severe effect but still decreased the EOP by more than 1,000-fold. Elimination of *merC*, however, had no effect on mercury resistance; the EOP of pNH6ΔC is not significantly different from that of pNH6, and the EOP of pNH6ΔPC is not significantly different from that of pNH6fsP. This result is consistent with the absence of *merC* in Tn*501*.

Extracts of cells containing the mutant plasmids had mercuric reductase activities (Table 4) that were not significantly lower than that of wild-type pNH6, showing that the Hg[s] phenotype of *merT* and *merP* mutants does not result from polar effects on *merA* and that mercuric reductase alone is not sufficient to confer resistance.

The high pI and low molecular weight of the *merP* gene product permit its visualization in whole-cell lysates by electrophoresis in cationic nondenaturing polyacrylamide gels at pH 7.0 (Fig. 5). The expected presence or absence of MerP was confirmed in all the mutant strains. The more slowly migrating band seen in the lane with the MerP standard is the reduced form of MerP (24a).

Phenotypes of mutants in an inducible, monocopy background. Because the *mer* phenotype is affected by plasmid copy number (38) and because *mer* occurs naturally on

TABLE 4. Mercuric reductase activity of *merTPC* mutants

Plasmid[a]	Hg(II) reductase activity (nmol of Hg · min^{-1} · mg of $protein^{-1}$)[b]	n
pTZ19R	0.07 ± 0.01	4
pNH6	5.8 ± 3.5	4
pNH6ΔT	4.2 ± 0.4	2
pNH6 fsP	1.9 ± 0.6	2
pNH6ΔC	7.9 ± 1.9	2
pNH6ΔTP	3.7 ± 0.7	2
pNH6ΔPC	5.1 ± 2.9	2
pNH6ΔTPC	3.0 ± 2.4	2

[a] All in *E. coli* JM103.
[b] Initial rates of ^{203}Hg volatilization from cell extracts containing 10 μM $^{203}HgCl_2$. Values are means ± standard errors of the means.

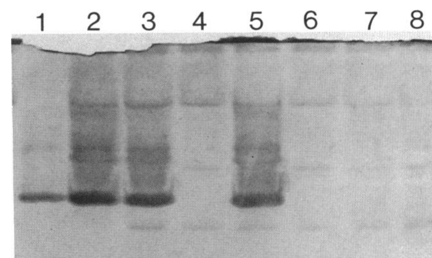

FIG. 5. Visualization of MerP protein in pNH6*merTPC* mutant lysates by nondenaturing PAGE. Lanes: 1, purified MerP protein; 2 through 9, lysates of JM103(pNH6) and mutant derivatives; 2, pNH6; 3, pNH6ΔT16; 4, pNH6fsP1; 5, pNH6ΔC37; 6, pNH6ΔTP37; 7, pNH6ΔPC1; 8, pNH6ΔTPC18. Electrophoretic migration is from top to bottom.

low-copy-number plasmids (47), we subcloned the *mer* region from pNH6 into the monocopy mini-F cloning vehicle pFZY1mp18 for additional physiological studies. Plasmid pNH8, the monocopy equivalent of pNH6, had a mercury-sensitive phenotype, since the microconstitutive level of expression is insufficient to produce mercury resistance (data not shown). However, when *merR* was provided in *trans* on the compatible plasmid pNH9, Hg(II) resistance equivalent to that of wild-type pDU202 was produced (Fig. 6). In the pNH8/pNH9 background, the effects of the mutations on Hg(II) resistance were more clearly seen by determining the EOP at several $HgCl_2$ concentrations, including 50 μM (Fig. 6). The *merP* mutation slightly decreased mercury resistance, the *merT* mutant was somewhat more impaired, and mutants deficient in both *merP* and *merT* were as sensitive as the vector. Again, elimination of *merC* did not impair Hg(II) resistance. These differences in resistance are reflected by the concentration of Hg(II) resulting in an EOP of <0.01: pNH8/pNH9 is inhibited at 100 μM; ΔC is inhibited at 200 μM; fsP is inhibited at 75 μM; ΔT is inhibited at 50 μM; and ΔTP, ΔTPC, and the pFZY1mp18 vector are all inhibited at 35 μM.

***mer* operon induction.** Inducibility and Hg(II) binding were assessed in pNH10, a mini-F derivative in which the carboxy-terminal portion of *merA* has been deleted beyond the *Eco*RI site (Fig. 2B). This construction puts *lacZ* under the control of the *mer* promoter, so that in the presence of pNH9 β-galactosidase activity is mercury inducible and provides a convenient measure of operon induction (Fig. 7). As expected for deletion of Hg(II) transport functions, the *merT* and *merP* mutants in the pNH10/pNH9 background required higher Hg(II) concentrations for maximal induction than did the $merT^+P^+C^+$ parent; the Hg(II) concentrations for 50% of maximal expression were between 0.2 and 0.4 μM for the *merT* and *merP* mutants, compared with approximately 0.07 μM for pNH10. The *merT* and *merP* mutants also increased the maximal expression of β-galactosidase by about 20 to 30%. For the *merTP* double mutant, induction of β-galactosidase did not plateau at the highest inducing concentration tested (1 μM); however, at least 0.5 μM Hg(II) was needed for 50% induction. The *merC* mutation (either alone or in combination) had little or no effect on the concentration of mercury required for half-maximal induction (0.1 μM for ΔC versus 0.07 μM for pNH10 and ≥0.5 μM for both ΔTP and ΔTPC) but consistently reduced the final amount of β-galactosidase activity by about one-third.

Mercury binding. Mutants lacking either *merT* or *merP* were deficient in Hg(II)-inducible Hg(II) binding (Fig. 8).

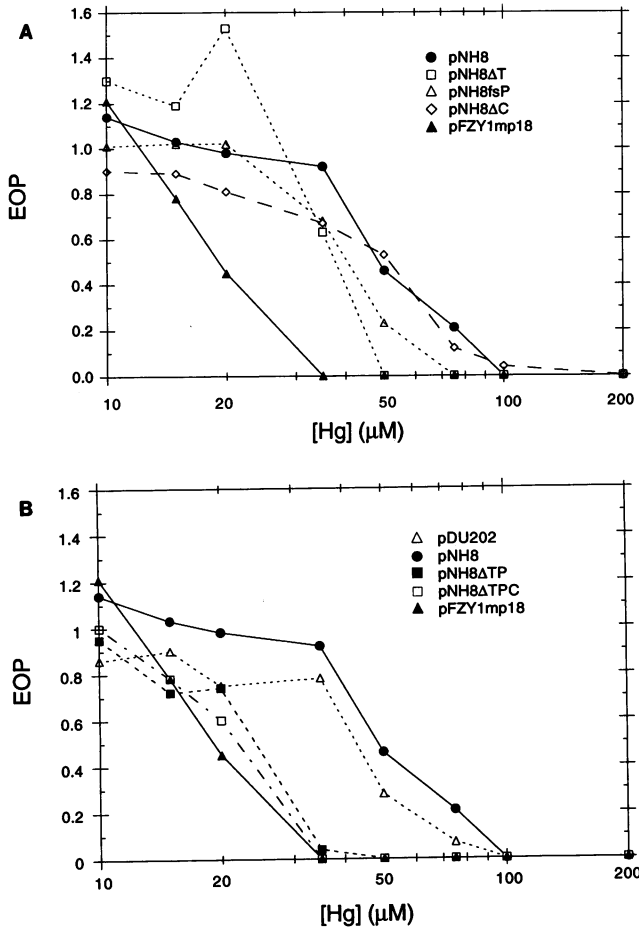

FIG. 6. Mercury resistance of *merTPC* mutants in a monocopy, inducible background. For pNH8 and its mutant derivatives in *E. coli* MC10615(pNH9), the EOP was determined by comparing the CFUs on L agar containing ampicillin, kanamycin, and various concentrations of $HgCl_2$ with the CFUs on L agar containing ampicillin and kanamycin. The values are means of four experiments.

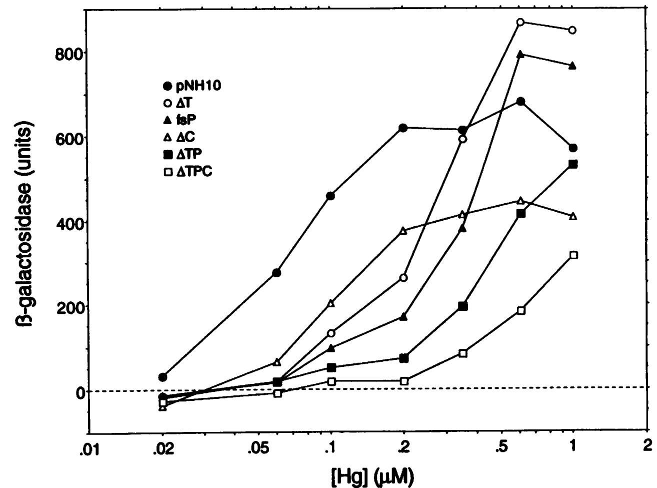

FIG. 7. Induction of *mer* expression in pNH10*merTPC* mutants as assessed by β-galactosidase activities of *mer-lac* operon fusions. Plasmid pNH9 was also present in all strains. The values are the means of 4 to 11 determinations.

Whereas pNH10 exhibits Hg(II)-inducible Hg(II) binding, the induced *merT*, *merP*, *merTP*, and *merTPC* mutants bind Hg(II) at a level that is indistinguishable from that of the vector control. (In all cases, the effectiveness of induction was monitored by assaying β-galactosidase.) In contrast, the mutant lacking only *merC* bound as much Hg as did the *merT*$^+$*C*$^+$*P*$^+$ parent. Furthermore, the lack of a detectable difference in binding between the *merTPC* mutant and the *merTP* mutant provides additional evidence that *merC* is not important in Tn*21* Hg(II) transport.

DISCUSSION

By constructing precise deletion and frameshift mutations in the *merT*, *merP*, and *merC* genes of the Tn*21 mer* operon, we dissected their individual roles in Hg(II) resistance and transport. Although previous studies (1, 2, 24, 26, 36, 38) have implicated the *merTPC* gene products in Hg(II) transport, the effect of loss of these genes from plasmids with an intact *merA* gene has been examined in only one previous study (26), in which *merR*, *merT*, *merP*, and the *mer* promoter were removed from Tn*501*. Although that study did show that the *merTP* region was required for Hg(II) resistance in Tn*501*, the conclusions were limited in that (i) quantitative conclusions about the importance of the transport genes could not be made because the strains compared were not otherwise isogenic, (ii) the roles of the individual genes were not examined, and (iii) there were no data on *merC*, since this gene is lacking in Tn*501*.

Our results establish that in Tn*21*, the *merP* and *merT* genes alone encode the Hg(II) transport system. In a *merA* mutant background, mutations in either or both of these genes abolished Hg(II)-inducible Hg(II) binding, whereas deletion of *merC* had no effect. This result is consistent with previous data (37) on Hg(II) uptake in Tn*21 merC*::Tn*5* insertions.

Both *merT* and *merP* were also needed for full Hg(II) resistance in both the high-copy-number plasmid pNH6, in which *mer* is expressed constitutively, and the monocopy plasmid pNH8, in which *mer* is inducible. Observing this result in the constitutive system shows clearly that the *merT* and *merP* gene products are intrinsically necessary for Hg(II) resistance, not simply for operon induction. This conclusion is also supported by the lowered resistance of a Tn*501 merRTP* deletion expressing *merA* from the chloramphenicol transacetylase promoter (26).

The deficits in resistance caused by the *merT* and *merP* mutations are manifested at Hg(II) concentrations much higher than those used to detect *mer*-mediated Hg(II) transport. This result argues against the conjecture that *merP* may "make transport and ultimate detoxification of mercuric ions much more efficient at low mercury concentrations" (3); we find that the decreased EOP of pNH6fsP is very marked at 50 μM Hg(II) (Table 3) and that the decreased EOP in pNH8fsP only occurs at >20 μM Hg(II) (Fig. 6). The fact that transport deficiency decreases resistance to high Hg(II) concentrations also emphasizes that measurement of *mer*-specific Hg(II) binding with Hg(II)-supersensitive *merA* mutants does not reflect normal *mer* Hg(II) transport function in *merA*$^+$ Hg(II)-resistant cells. When MerA is functional, intracellular Hg(II) concentrations are greatly diminished by rapid volatilization, both decreasing toxicity and increasing the concentration gradient of Hg(II). Thus, mercuric acid

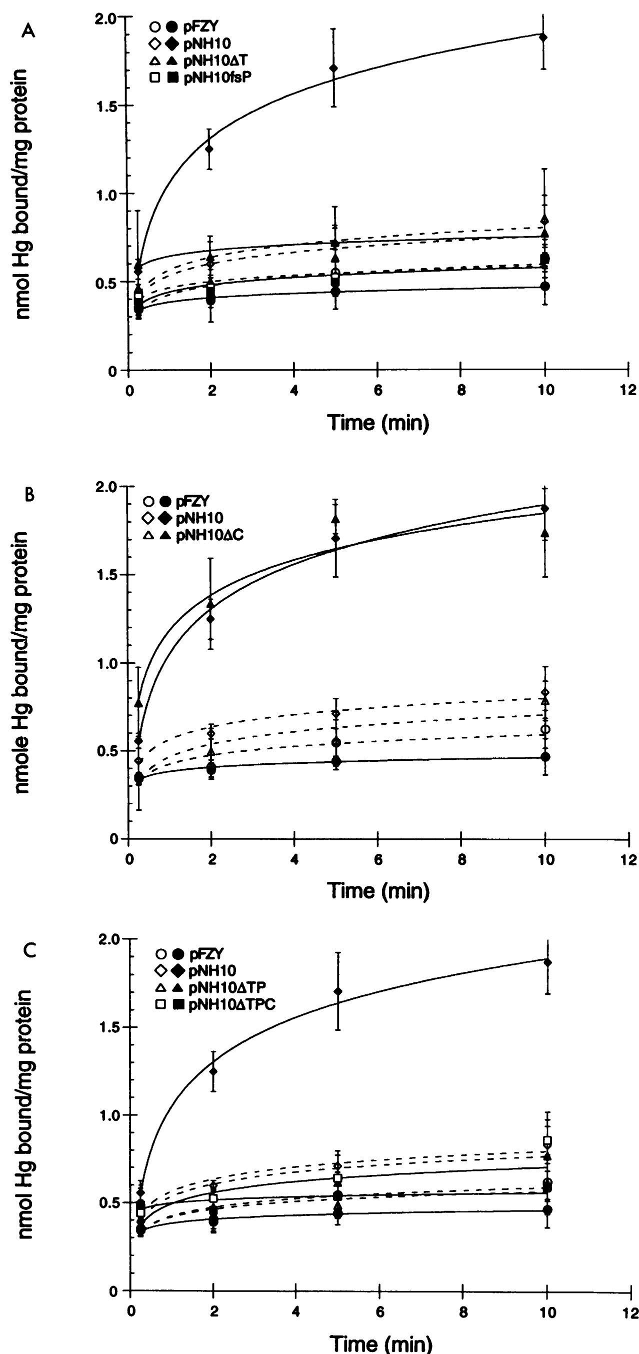

FIG. 8. Binding of ^{203}Hg by *E. coli* MC1061-5 carrying pNH9 (*merR*$^+$) and monocopy *merTPC* (pNH10) derivatives. Symbols: ○, ◇, △, □, uninducted; ●, ◆, ▲, ■, Hg induced. The values are means of three experiments. The error bars show the standard deviations.

reductase allows Hg(II) transport to operate at external Hg(II) concentrations higher than those that can be used to measure transport in *merA* mutants (50).

In contrast to the indistinguishable effects of single *merT* and *merP* mutations on Hg(II) binding, the *merT* mutation decreased Hg(II) resistance more than the *merP* mutation; furthermore, mutations eliminating both *merP* and *merT* caused more drastic effects on Hg(II) resistance than did the single mutations. This decreased resistance is most clearly seen in the monocopy inducible background (Fig. 6). If MerT and MerP function only as sequential components in an Hg(II) transport pathway, mutations in either should have the same effect and mutants defective in both genes should have the same phenotype as mutants defective in the individual genes. Thus, the distinct resistance phenotypes and the additive effect of *merT* and *merP* mutations suggest that they may function at least partially independently in providing Hg(II) resistance. It has been suggested, for instance, that MerP may act as a sponge (47), scavenging Hg(II) from the periplasmic space and thereby "protecting the periplasmic constituents and the outer face of intrinsic inner membrane proteins from Hg(II)" (3). MerT might have a somewhat analogous role in protecting the cytoplasm. If MerA interacts directly with MerT, as has been proposed (3, 32, 47), MerT might remove Hg(II) from cytoplasmic target proteins and transfer it to the reductase. Thus, MerT could still facilitate detoxification of Hg(II) that has entered the cell nonspecifically.

Extracts of cells bearing the *merT* or *merP* mutant plasmids can still volatilize Hg(II), indicating that their effect on Hg(II) resistance does not result from transcriptional polarity on *merA*. The normal levels of mercuric reductase in these strains emphasize that this enzyme alone cannot confer resistance and that it must work in concert with the transport system for its potential to be realized.

Both the *merT* and *merP* mutations affected induction of β-galactosidase activity in *merA-lacZ* transcriptional fusions. It has been known since the pioneering work on lactose permease mutants (44) that if an inducer enters the cell via a transport system, transport-deficient mutants will fail to induce. We wanted to determine not only whether these mutations caused this effect but also whether they exhibited any additional regulatory anomalies, especially since *merC* had previously been implicated in regulation. The effects of *merT* or *merP* mutations on induction varied as a function of Hg(II) concentration; β-galactosidase could be induced in the *merT* or *merP* mutants, but a higher concentration of Hg(II) was required for induction, as expected for Hg(II) transport deficiency. Thus, in order to exhibit its optimum response in whole cells, the MerR Hg(II) sensor requires an intact Hg(II) transport system; however, our data do not indicate any specific regulatory function for MerT and MerP. Our results are consistent with the previous finding that a *merTP* deletion in Tn*501* [assayed at a single Hg(II) concentration] decreased operon induction (26). The *merC* deletion only slightly increased the minimal inducing concentration of Hg(II), consistent with its lack of effect on Hg(II) binding.

Both the *merT* and *merP* mutations resulted in greater maximum expression of β-galactosidase in the *merA-lacZ* transcriptional fusions, whereas the *merC* deletion decreased expression. We cannot tell whether these effects are caused by absence of the protein products or by altered regulatory sites in the DNA, although the fact that the *merP* and *merT* mutations do not overlap makes it unlikely that the same regulatory site is affected in both mutants. Many Tn*21* *mer* mRNA molecules terminate near the *merA-C* junction, producing decreased expression of *merAD* (14). The effects of our mutations on expression may have resulted from altering these premature terminations, although we would have then expected the *merC* mutation to increase expression of the *merA-lacZ* fusion. Clearly further work is needed to understand the role of *merTPC* in operon expression.

The role of *merC* in Tn*21* remains a puzzle. In *T. ferrooxidans* a gene homologous to *merC* encodes an Hg(II) transport protein with relatively weak activity (24). The Tn*501* *mer* operon, however, lacks a *merC* gene but nonetheless has a functional Hg(II) binding system and provides Hg(II) resistance equivalent to that of Tn*21*. Our results provide evidence that *merC* does not have a role in Hg(II) transport or in resistance in the Tn*21* *mer* operon. These conclusions are consistent with the observations with Tn*501* but raise the question of whether *merC* has any function in Tn*21*. However, several studies (15, 42, 43) show that the *merC* gene is widely distributed in nature, suggesting that it may have a role we have yet to determine.

ACKNOWLEDGMENTS

We thank Paul Totis, Melanie Craft, and Mary Homer for technical assistance.

This work was supported by National Science Foundation grants DMB-8508688 and DMB-8705101 to N.V.H. and by Public Health Service grant GM28211 from the National Institutes of Health to A.O.S.

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